Original Article

High permeability haemofiltration improves peripheral blood mononuclear cell proliferation in septic patients with acute renal failure

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Abstract

Background. Continuous veno-venous haemofiltration (HF) with high permeability (HP) haemofilters is a novel approach in the adjuvant therapy of septic patients. HP haemofilters are characterized by an increased pore size which facilitates the filtration of inflammatory mediators. The present study examines whether HP-HF has an impact on peripheral blood mononuclear cell (PBMC) proliferation and whether ultrafiltrate can alter PBMC function in isolates from healthy volunteers.

Methods. Twenty-eight septic patients with acute renal failure were randomly allocated to either HP-HF or conventional HF (C-HF). HP-HF was performed with a newly developed high-flux polyamide membrane (P2SH) with a nominal cut-off point of 60 kDa. For C-HF, a high-flux polyamide haemofilter (Polyflux 11S; cut-off, 30 kDa) was used.

Results. Septic patients demonstrated a significantly reduced proliferation of anti-CD3-stimulated PBMCs compared to healthy controls (P = 0.016). Initiating HF led to a restoration of the PBMC proliferation in HP-HF but not in C-HF. Exposing PBMCs isolated from healthy donors to ultrafiltrates from patients with sepsis demonstrated a significant suppressive effect of HP ultrafiltrates on the anti-CD3-stimulated PBMC proliferation (P = 0.011). Ultrafiltrate from patients with sepsis who received C-HF had no impact on PBMC proliferation.

Conclusion. HP-HF restores PBMC proliferation in septic patients probably by eliminating immunomodulatory mediators. HP-HF may represent a new renal replacement therapy able to modulate PBMC function in sepsis.

Keywords: acute renal failure; high permeability haemofiltration; immunomodulatory mediators; PBMC proliferation; sepsis

Introduction

The multiple organ dysfunction syndrome (MODS) is the most common cause of acute renal failure (ARF) in the intensive care unit. In many cases, MODS is a complication of severe infection, sepsis or septic shock. Although the understanding of its pathophysiology remains incomplete, there is evidence that sepsis leads to a significant depression in T-lymphocyte-mediated immunity [1,2].

In contrast to the non-specific immunity of the granulocytes, lymphocytes constitute the specific component of cell-mediated immunity which becomes activated in response to antigen processing. The proliferative capacity of lymphocytes has proven to be reduced during sepsis. Toft et al. [2] analysed the phytohaemagglutinin (PHA) mitogen stimulated lymphoproliferative response following infusion of endotoxin in a sepsis animal model and observed a significant depression of lymphoproliferative activity. This is in line with findings made by Meldrum et al. [1] who reported a reduced lymphoproliferative response to PHA stimulation, following endotoxin administration. Muret et al. [3] recently described a reduced lymphoproliferative response in SIRS patients. Heidecke et al. [4] found a significantly suppressed T-lymphocyte proliferation in patients with lethal intra-abdominal infection, but not in survivors. He concluded that
HP-HF improves PBMC proliferation

immune defects that are intrinsic to T cells and are characterized by an anergic lymphocyte proliferative phenotype are associated with lethality of intra-abdominal infection.

The aim of this study was to analyse the impact of high permeability haemofiltration (HP-HF) on lymphocyte proliferation in septic patients with ARF. For this purpose, peripheral blood mononuclear cells (PBMCs) were isolated and lymphocyte proliferation was stimulated by using monoclonal anti-CD3 antibodies. Baseline values were compared with follow-up values at 60 h after initiating HF. Patients treated with conventional HF (C-HF) served as controls. In addition, we characterized the specific qualities of ultrafiltrate by exposing septic ultrafiltrate to PBMCs isolated from healthy volunteers.

HP-HF is an emerging new HF modality designed to facilitate the elimination of inflammatory mediators. HP-HF is performed with specially designed haemofilters characterized by an increased pore size. In a lethal animal model of sepsis, HP-HF was shown to prolong survival [5]. We reported about the safety of this procedure in clinical settings and provided the first evidence on the superiority of HP-HF with regard to the elimination of inflammatory cytokines compared with HF performed with conventional haemofilters [6].

Subjects and methods

Study population

The study was approved by the Ethical Committee on Human Research of the University Hospital Charité of Berlin and was in accordance with the Declaration of Helsinki. Twenty-eight critically ill patients were randomly allocated to either HP-HF or C-HF. ARF was diagnosed by the nephrology staff on clinical grounds. Sepsis was diagnosed as defined by the ACCP/SCCM Consensus Conference [7].

Haemofiltration procedure

All patients were treated with continuous veno-venous HF systems (BM 25; Baxter Healthcare, Munich, Germany). HF was initiated with the onset of ARF in the course of a septic shock. ARF was diagnosed as follows: (i) fluid overload owing to inadequate urine production despite administration of diuretic agents and maintenance of adequate blood pressure; (ii) a rise in serum creatinine above 2.5 mg/dl or a doubling of the creatinine from baseline; (iii) serum potassium above 5.5 mmol/l due to oligo-anuria.

The study period was restricted to 60 h. HP-HF was performed in alternation with C-HF for a daily 12 h period. A total of three HP-HF courses were thus performed during the study period.

HP-HF was performed with a newly developed high-flux polyamide™ haemofilter (P2SH, effective surface area 0.6 m², steam sterilized, cut-off point ~30 kDa; Gambro Corporate Research, Hechingen, Germany). The membrane for HP-HF is characterized by an increased pore diameter leading to a higher permeability for substances in the molecular weight range up to 60 kDa. C-HF was performed with a commercially available standard high-flux polyamide™ membrane (Polyflux 11 S, effective surface area 1.1 m², steam sterilized, cut-off point ~30 kDa; Gambro Dialysatoren, Hechingen, Germany). The haemofilter was changed daily in the C-HF group. Blood flow rate was 150 ml/min. The HF replacement volume was 24 l/day for all patients throughout the study period. Fluids were added in the post-dilutional mode. The extracorporeal circuit was anticoagulated with continuous unfractionated heparin infusion in an individual patient-adjusted anticoagulation regime.

Haemofiltration in healthy volunteers. As controls, short time (30 min) HP and C-HF was performed in three healthy volunteers. Vascular access was obtained with two dialysis needles (15-gauge) inserted into the basilic vein of the right and left arm (to exclude recirculation). To prevent filter clotting, 3000 IU of unfractionated heparin was given into the extracorporeal circuit. Blood flow was maintained at 120 ml/min throughout the experiment. The ultrafiltration volume was set at 1 l/h, replacement fluids were added in the post-dilutional mode. After 30 min of C-HF, filtrate was taken and immediately stored at −80 °C until assay. Then, the haemofilter was changed to the HP haemofilter and after 30 min, filtrates were sampled again.

Isolation of peripheral blood mononuclear cells

PBMCs were isolated by Ficoll density centrifugation as previously described [8]. Briefly, 10 ml of citrate anticoagulated blood were diluted with 10 ml of Dulbeco’s phosphate buffered saline (PBS; PAA laboratories, Linz, Austria). Then, 4 ml of Ficoll (Ficoll-Paque™ Plus; Amersham Pharmacia Biotech, Uppsala, Sweden) were overlaid with 10 ml of blood/PBS. After centrifugation (MegaFuge-2.0R; Heraeus Instruments GmbH, Berlin, Germany) for 20 min at 1650 g, PBMCs were collected from the interface between the plasma and the Ficoll layer, and washed with PBS and dissolved in RPMI-1640 medium supplemented with 10% FCS, 100 U of penicillin/streptomycin (100 mg/ml) and 2 mM glutamine (Biochrom, Germany). PBMCs were seeded in 96-well plates at a final concentration of 1 × 10⁵ cells/well. Cell viability was determined by trypan blue staining (Sigma Aldrich Chemie GmbH, Steinheim, Germany).

PBMC proliferation assay

To specifically stimulate the proliferation of lymphocytes, PBMCs were stimulated with 10 ng/ml monoclonal anti-CD3 antibodies (murunonab-CD3; Ortho Biotech Product, L.P., Raritan, NJ, USA). The 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide (MTT) test was used to assess cell proliferation as described previously [9,10]. Briefly, PBMCs (10⁵ cells/ml) were cultured in RPMI medium (Biochrom) in humidified air at 37 °C, 5% CO₂, for 72 h. Then, MTT solution (20 μl/5 mg per ml) (Sigma, Steinheim, Germany) was added to 100 μl of cell suspension in 96-well plates (BD-Falcon, New Jersey, USA) and incubated for 24 h at 37 °C. The formazan crystals were dissolved with 10% SDS in 0.01 N HCl solution (Merck, Darmstadt, Germany) and the absorbance was measured by an ELISA (Dynatech, Guersey, Channel Islands) reader at 570 nm.

Titration curves were performed prior to the experiments in order to standardize and optimize the MTT proliferation...
assay. We found a linear dose (anti-CD3)-dependent proliferation of PBMCs with a maximal effect at 10 ng/ml anti-CD3 (10⁶ cells/ml). In addition, kinetics revealed a maximum stimulation after 3 days (data not shown). Ten healthy volunteers donated blood for characterizing the PBMC proliferation of healthy volunteers. Blood samples were taken twice at baseline (t = 0) and after 60 h (t = 60).

To analyse the effects of ultrafiltrates, PBMCs were isolated from six healthy volunteers as described above. Then, filtrates (50 μl) were incubated with the cell suspension (50 μl) for the whole assay period. Filtrates taken at 30 min after initiation of HF (either HP-HF, C-HF or healthy controls) were used for the ultrafiltrate experiments. The final cell concentration of the assay was 1 x 10⁵ cells/well. PBMC proliferation was measured as outlined above.

All experiments were done in quadruplicate. Intra-test standard deviation was <5%. The PBMC viability was above 95% throughout the whole experiment.

Statistical analysis

After testing for normal distribution (Levene test) the Kruskal–Wallis H-test was performed to compare multiple independent samples (control, C-HF, HP-HF) for each time period (t = 0 and 60).

Paired analyses were performed to detect differences in the proliferative activity over time (t = 0–60 h). After testing for normal distribution, the Wilcoxon rank sum test was used for non-parametric data and the Student’s t-test for parametric data. Results were expressed as the median with the interquartile range. A P-value < 0.05 was considered significant. The statistical program SPSS (SPSS Inc., Chigaco, IL, USA) was used for analysis. The graphical presentation of the data was done by box and whisker plots. The boxes show the median values and the 25th and 75th percentiles, and the extremes are represented by the whiskers.

Results

Patients

The clinical characteristics are summarized in Table 1. Baseline clinical data did not differ between both groups. Pneumonia was the most prevalent cause for sepsis (n = 12), followed by bacterial peritonitis (n = 4), urosepsis (n = 4), necrotizing pancreatitis (n = 3), mediastinitis after coronary bypass surgery (n = 2), bacterial endocarditis (n = 1), cholecystitis (n = 1) and catheter infection (n = 1). All patients required mechanical ventilation and were on vasopressive support throughout the study period.

Table 1. Clinical characteristics of the study population prior to the initiation of HF

<table>
<thead>
<tr>
<th></th>
<th>HP-HF (n = 14)</th>
<th>C-HF (n = 14)</th>
<th>P-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age (years)</td>
<td>66 (58–73)</td>
<td>65 (58–70)</td>
<td>NS</td>
</tr>
<tr>
<td>Gender (male/female)</td>
<td>10/4</td>
<td>11/3</td>
<td>NS</td>
</tr>
<tr>
<td>APACHE-II score</td>
<td>28.5 (21.0–30.8)</td>
<td>26.7 (23.0–34.5)</td>
<td>NS</td>
</tr>
<tr>
<td>MODS score</td>
<td>6.0 (4–8)</td>
<td>7.0 (6–8.2)</td>
<td>NS</td>
</tr>
<tr>
<td>Albumin (mg/dl)</td>
<td>2.61 (2.2–2.7)</td>
<td>2.7 (2.3–2.9)</td>
<td>NS</td>
</tr>
<tr>
<td>Bilirubin (mg/dl)</td>
<td>1.2 (0.8–2.3)</td>
<td>2.1 (0.9–3.5)</td>
<td>NS</td>
</tr>
<tr>
<td>Leucocytes (10⁵/μl)</td>
<td>13.4 (9.8–25.8)</td>
<td>17.5 (11.8–25.9)</td>
<td>NS</td>
</tr>
<tr>
<td>Prothrombin time (%)</td>
<td>75 (58–86)</td>
<td>68 (51–91)</td>
<td>NS</td>
</tr>
<tr>
<td>Creatinine (mg/dl)</td>
<td>2.1 (1.4–3.8)</td>
<td>2.2 (1.3–2.7)</td>
<td>NS</td>
</tr>
<tr>
<td>Urea (mg/dl)</td>
<td>156 (94–192)</td>
<td>142 (102–185)</td>
<td>NS</td>
</tr>
<tr>
<td>PaO₂/FIO₂</td>
<td>199 (154–383)</td>
<td>266 (198–378)</td>
<td>NS</td>
</tr>
<tr>
<td>Norepinephrine (µg/kg/min)</td>
<td>0.32 (0.04–0.60)</td>
<td>0.31 (0.28–0.60)</td>
<td>NS</td>
</tr>
</tbody>
</table>

The APACHE-II score was measured on the basis of the data assessed on the ICU admission day. The MODS score was conservatively scored at enrolment into the study.

Proliferative activity of PBMCs isolated from septic patients

The spontaneous (unstimulated) proliferative activity of PBMCs isolated from septic patients immediately prior to HF did not differ between the C-HF and the HP-HF group (Figure 1A). Furthermore, the unstimulated proliferative activity of isolated PBMCs from septic patients was comparable with that of healthy volunteers (Figure 1A). At 60 h of HF a significant increase in the spontaneous PBMC proliferation of septic patients treated with HP-HF was observed (P = 0.019 vs baseline). Values remained unchanged in the C-HF group (Figure 1A and B).

The proliferative activity of anti-CD3 antibody-stimulated PBMCs prior to HF was reduced in all septic patients compared with healthy controls. Although values did not reach statistical significance in a multivariate approach (P = 0.056), pooling the septic data and comparing septic (n = 28) vs healthy controls (n = 10) revealed a highly significant difference in the anti-CD3-stimulated PBMC proliferation (P = 0.016; Figure 2A).

Initiating HF led to recovery of PBMC proliferative activity in the HP-HF group, whereas values remained unchanged during C-HF (Figure 2A and B). At 60 h of HF, anti-CD3-stimulated PBMC proliferation was significantly higher in the HP-HF group compared with the C-HF group (P = 0.011; Figure 2B).

Effect of ultrafiltrate on PBMC proliferation

The effects of ultrafiltrate on the proliferation of healthy PBMCs were analysed under unstimulated and anti-CD3-stimulated conditions (Table 2).
HP-HF filtrate from septic patients had a depressant effect on anti-CD3-stimulated PBMCs, and a marginal effect (\(P = 0.051\)) on spontaneous PBMC proliferation. There was an important difference between the effects of filtrates from patients with sepsis obtained by C-HF vs HP-HF on spontaneous, and anti-CD3-stimulated PBMC proliferation.

To exclude unspecific effects of the filtrate we compared the spontaneous PBMC proliferation of the three healthy volunteers (not exposed to filtrate) with the proliferation rate of PBMCs challenged with conventional septic and healthy filtrates and found no statistical differences. Proliferation values from the three volunteers were 0.18 [interquartile range (IQR)
0.17–0.19; \( P = 0.73 \) vs HP-HF filtrates from healthy volunteers] for the spontaneous and 0.65 (IQR 0.39–0.71; \( P = 0.14 \) vs HP-HF from healthy volunteers) for the anti-CD3-stimulated assay.

### Discussion

Functional alterations of the adaptive immune system during severe illness are considered important for the development of sepsis. Lymphocytes constitute the specific component of cell-mediated immunity which becomes activated in response to antigen processing. When activated, lymphocytes proliferate and differentiate to their effector cells promoting T-cell-mediated cytotoxicity and antigen presentation or B-cell-mediated immunoglobulin secretion. The proliferative capacity of lymphocytes is reduced during sepsis and correlates with increased mortality [4]. A well-established procedure to analyse lymphocyte proliferation is to isolate PBMCs and to specifically stimulate lymphocyte proliferation by adding anti-CD3 antibodies to the assay.

We observed a significantly reduced proliferation in anti-CD3-stimulated PBMCs in all septic patients compared with PBMCs isolated from healthy controls. Even the spontaneous PBMC proliferation rate, which was expected to be increased given the circumstances of severe sepsis, was found to be within the normal range. Both the normal spontaneous proliferation rate of PBMCs during sepsis as well as the reduced proliferation capacity during anti-CD3 stimulation indicate a depression of PBMC function.

However, the key finding of this study was that initiating HF led to a restoration of the PBMC proliferation capacity in the HP-HF but not in the C-HF group. The restoration of a ‘normal’ proliferation activity during anti-CD3 challenge may be explained by the induction of a higher spontaneous PBMC proliferation rate during HP-HF.

We went further and characterized the specific qualities of ultrafiltrate by exposing isolated PBMCs from healthy volunteers to ultrafiltrates and measuring cell proliferation. Filtrates from HP-HF, but not from C-HF, had a suppressive effect on the spontaneous (unstimulated) and the anti-CD3-stimulated PBMC proliferation. In addition, filtrates taken from healthy volunteers who underwent both HP-HF and C-HF did not differ from conventional septic filtrates in their PBMC proliferation capacity. Our filtrate experiments, thus, give evidence that immunomodulatory factors are eliminated by HP-HF which usually cannot be eliminated by C-HF.

In the literature, only a few data are available on the impact of CVVH or CVVHD on PBMC proliferation. Toft et al. [2] showed that CVVHD per se has no impact on the lymphoproliferative response. Hoffmann et al. [9] demonstrated that septic ultrafiltrates taken from C-HF did not alter the proliferation of PBMCs isolated from healthy volunteers. Reproducible alteration in PBMC proliferation only occurred when filtrates were incubated with endotoxin. Nevertheless, lymphocyte cultures stimulated with PHA demonstrated a significant decrease in IL-2 and IL-6 release when incubated with septic filtrates.

The mediators for T-lymphocyte suppression are yet not completely elucidated. It is generally accepted that sepsis leads to a significant depression in T-cell-mediated immunity, which is associated with an increased release of both inflammatory and anti-inflammatory mediators such as prostaglandin E2 (PGE2), transforming growth factor-\( \beta \) (TGF-\( \beta \)), interleukin (IL)-6, IL-4 and IL-10 [10].

In addition, there is a shift from T-helper-1 to T-helper-2 lymphokines, which leads to a decrease in IL-2 and interferon-gamma production and an increase in IL-4 and IL-10 formation [10–12]. The upregulation of IL-4, IL-10 and TGF-\( \beta \) production has been shown to have a role in the decrease in IL-2 release and the suppression of lymphocyte proliferation during sepsis [10,13,14]. In addition, several studies have proven that increased PGE2 production inhibits lymphocyte proliferation [15,16].

Whether the elimination of inflammatory mediators or other blood compounds, such as prostaglandins or TGF-\( \beta \), are responsible for the beneficial effects on

### Table 2. Impact of ultrafiltrates on PBMC proliferation in vitro

<table>
<thead>
<tr>
<th>Filtrate from healthy volunteers (( n = 3 ))</th>
<th>Filtrate from patients with sepsis (( n = 14 ))</th>
</tr>
</thead>
<tbody>
<tr>
<td>C-HF</td>
<td>HP-HF</td>
</tr>
<tr>
<td>Spontaneous PBMC proliferation</td>
<td>0.18 (0.11–0.20)</td>
</tr>
<tr>
<td>Anti-CD3-stimulated PBMC proliferation</td>
<td>0.44 (0.36–0.46)</td>
</tr>
</tbody>
</table>
PBMC proliferation seen in our study remains to be clarified.

Continuous veno-venous HF constitutes a reliable extracorporeal renal replacement therapy for the treatment of ARF. It might also be beneficial as an adjuvant treatment for septic shock, through a non-specific extracorporeal removal of inflammatory cytokines [17,18]. Although pre-clinical studies have yielded encouraging results, recent human studies have failed to prove effectiveness in vivo [17,18].

The limited removal of inflammatory mediator by C-HF is due to the relatively low cut-off point of the applied membranes. Commercially available HF membranes achieve good clearances for smaller middle molecules, such as β2 microglobulin which has a molecular weight of 11.8 kDa [19]. The cut-off point of these membranes, defined as a sieving coefficient below 5% (SC = 2 × ultrafiltrate concentration of the molecule/concentration of the molecule at the filter inlet + concentration at the filter outlet), lies around 30 kDa, as is the case in our conventional polyamide membrane. The smaller the molecule with regard to the cut-off point of the membrane the better its elimination. Electric charges, molecular structure, hydrophilicity and hydrophobicity, as well as filtration modalities such as the transmembrane pressure are important additional determinants for the efficacy of compound filtration [19].

Adsorption of inflammatory mediators to the haemofilter has been found to represent an additional elimination pathway in CRRT. We cannot comment on mediator adsorption in our study and its impact on PBMC proliferation. The fact that the C-HF group had less filter changes (during the study period a total of five filters were used in the HP-HF group compared with only three filters in the C-HF group) could have theoretically led to a bias in favour of the HP-HF group. However, we do not believe that adsorption plays a role in our setting for two reasons. First, adsorption is dependent on the membrane characteristics of the haemofilter. Polyamide membranes have been found to have a poor adsorptive capacity [19,20]. Secondly, saturation of the membrane usually occurs within a few hours or even minutes [18]. In our HP-HF setting membranes were changed twice daily so that the impact on circulating mediators could be expected to be poor.

HP haemofilters are characterized by a significantly increased cut-off point which is achieved by an increase in effective pore size of the membrane. Although pore size decreases over time due to blood compound deposition, effective clearance capacity of middle-sized molecules in HP-HF remains superior to C-HF [6]. We recently reported that the cut-off point of our membrane rapidly declines to ~50 kDa where it remains stable over time [6]. Protein loss, a side effect of HP-HF, was not excessive and had no impact on blood protein levels. The increased pore size allowed a substantial filtration of inflammatory IL-6 which has a molecular weight of 28 kDa. However, the elimination capacity of TNF-α (51 kDa) was poor. Uchino et al. [20] tested the same membrane in an in vitro approach were he could reproduce our findings on IL-6 and TNF-α elimination. In addition, he observed very good clearances also for IL-1β, IL-8 and IL-10.

We are aware that our study has limitations. C-HF has been performed with a dose of 15 ml/kg/h which is a low treatment dose. We cannot comment on whether high volume C-HF might be able to achieve the same results as HP-HF. However, our filtrate experiments show that conventional filtrates do not influence PBMC proliferation. In fact, conventional filtrates were comparable with filtrates sampled from healthy controls who performed short-term C-HF. Increasing the HF volume from 14 to 35 ml/kg/h may increase the overall clearance capacity but does change the composition of the filtrate.

However, further trials including more patients are necessary to evaluate whether HP-HF has a beneficial effect on the clinical course of septic patients.

Conflict of interest statement. None declared.

References


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